FORM PTO-1390 (REV. 11-94)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)		8484-075-999 09/423712

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE98/01306	8 May 1998	9 May 1997

TITLE OF INVENTION
TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

APPLICANT(S) FOR DO/EO/US
Nawroth et al.

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). - Unexecuted

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information: Preliminary Amendment; Copy of Claims as amended under Article 34.; Statement Accompanying Substitute Specification.

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE
PCT/DE98/01306 09/427712	8 May 1998

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	31 - 20	11	X $ 18.00	$ 198.00
INDEPENDENT CLAIMS	6 - 3	3	X $ 78.00	234.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 260.00	$ 260.00

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY** ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $ 670	
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... $ 760	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) ..... $ 96	
☒ Filing with EPO or JPO search report ..... $ 840	$ 840.00
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).	
TOTAL OF ABOVE CALCULATIONS	= 1,532.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).	- $ 0.00
SUBTOTAL	= 1,532.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).	+
0 TOTAL FEES ENCLOSED	$ 1,532.00

a. ☐ A check in the amount of $__ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $ 1,532.00.00 to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☒ Other instructions

Please enter the Preliminary Amendment prior to calculating the fees.

19. ☒ All correspondence for this application should be mailed to

PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

20. ☒ All telephone inquiries should be made to (212) 790-2803

NAME	SIGNATURE	REGISTRATION NUMBER	DATE
Laura A. Coruzzi	Laura A. Coruzzi by: Reg No 43,341	30,742	11/09/99

Express Mail No. EL 451 593 119 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Nawroth *et al.*

Serial No.: UNASSIGNED

Group Art Unit: UNASSIGNED

Filed: HEREWITH

Examiner: UNASSIGNED

For: TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

Attorney Docket No.: 8484-075-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicant respectfully requests entry of the following amendments prior to examination of the above-referenced application.

AMENDMENTS

IN THE CLAIMS:

Please amend the claims as follows:

1. (Amended) [Use of tissue factor or a fragment thereof for the well-calculated therapeutic influence of] A method for modulating blood vessel formation in a subject in need, comprising [by induction of a local expression of a tissue factor nucleic acid or by local application of] locally administering a functional tissue factor protein in a therapeutically effective amount to said subject in need..

2. (Amended) [Use according to claim] The method of Claim 1 or 20, wherein [the influence is an activation of] said modulating is an activation of blood vessel formation.

3. (Amended) [Use of a tissue factor or a fragment thereof for influencing the] A method for enhancing wound healing in a subject in need, comprising [by induction of a local expression of a tissue factor nucleic acid or by local application of] locally administering a functional tissue factor protein in a therapeutically effective amount to said subject in need.

4. (Amended) [Use according to claim] The method of Claim 3 or 21, wherein the said subject in need is afflicted with [wound healing in the case of] diabetis mellitus, vasculitis, arterial conclusive disease, chronic venous and infected ulcer, innervation impairment, decubital gangrene [and] or weak sutures [in the case of operations is concerned] after a surgery.

5. (Amended) [Use according to claim 1 or] The method of Claim [1 or] 2, wherein [the blood vessel formation in the case of] said subject in need is afflicted with arteriosclerosis, Crohn's disease [and], ulcerative colitis, diabetic retinopathy [and], or deep venous thrombosis of the legs/ulcus cruris [is concerned].

6. (Amended) [Use according to claim 1 or] The method of Claim 2, wherein the blood vessel formation [for replacing] is activated for the replacement of impaired blood vessels [is concerned].

7. (Amended) [Use according to any one of claims 1 to 6] The method of Calim 2, wherein the tissue factor or a fragment thereof is present as expressible nucleic acid.

8. (Amended) [Use according to claim] The method of Claim 7, wherein [the expression of the] said nucleic acid is [transient] expressed transiently.

9. (Amended) [Use according to claim] The method of Claim 7 [or 8], wherein [the] said nucleic acid is a DNA.

10. (Amended) [Use according to any one of claims] The method of Claim 7 [to 9], wherein [the] said nucleic acid is controlled by a constitutive or an indiciuble promoter.

11. (Amended) [Use according to any one of claims] The method of Claim 7 [to 10], wherein the nucleic acid is present in a Sindbis virus replicon vector.

12. (Amended) [Use according to any one of claims] The method of Claim 7 [to 10], wherein the nucleic acid is controlled by a CMV or 5V40 promoter.

13. (Amended) [Use according to any one of claims] The method of Claim 1 [to 12], 3, 20, or 21, wherein the tissue factor is present in a liposome or on a carrier, particularly gold particle.

14. (Amended) [Use according to any one of claims 1] The method of Claim 13, wherein the tissue factor is present in combination with further factors promoting the formation of blood vessels.

15. (Amended) [Use according to claim] The method of Claim 14, wherein [the] said further factors are present as expressible nucleic acids or functional proteins.

16. (Amended) [Use according to claim 14 or] The method of Claim 15, wherein [one of the factors] at least one further factor is present and said further factor is VEGF.

17. (Amended) [Use according to any one of claims] The method of Claim 1 [to 16], 3, 20, or 21, wherein the tissue factor is present in a pharmaceutical composition.

Please add the following new Claims 18-21:

18. (New) A pharmaceutical composition for modulation of blood vessel formation, comprising tissue factor or a fragment thereof and a pharmaceutically acceptable carrier.

19. (New) A pharmaceutical composition for activation of blood vessel formation, comprising tissue factor or a fragment thereof and a pharmaceutically acceptable carrier.

20. (New) A method for modulating blood vessel formation in a subject in need, comprising inducing local expression of a tissue factor nucleic acid in said subject in need thereof.

21. (New) A method for enhancing wound healing in a subject in need, comprising inducing local expression of a tissue factor nucleic acid in said subject in need.

REMARKS

The above-made amendments do not introduce new matter and are fully supported by the specification and the claims as originally filed. Applicant respectfully requests that the amendments be made of record in the file history of the instant application.

The Commissioner is authorized to charge any underpayment and credit any overpayment to Pennie & Edmonds Deposit Account No. 16-1150. A copy of this sheet is provided.

Respectfully submitted,

Date November 9, 1999

Reg. No. 43,341
Birgit Millauer, Ph.D.

Laura A. Coruzzi Reg. No.30,742
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Nawroth *et al.*

Group Art Unit: Not Yet Known

Serial No.: Not Yet Known

Examiner: Not Yet Known

Filed: Herewith

Attorney Docket No.: 8484-075-999

For: TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

STATEMENT ACCOMPANYING SUBSTITUTE SPECIFICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Pursuant to 37 C.F.R. § 1.125, submitted herewith is a Substitute Specification. The Substitute Specification differs from the English translation of the original German Specification in that it has been reformatted to comply with U.S. practice. The Substitute Specification does not contain new matter. Entry of the substitute specification is kindly solicited.

No fee is believed due. However, if it is determined that additional fees are due, please charge them to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed for accounting purposes.

Respectfully submitted,

Date: November 9, 1999

Laura A. Coruzzi 30,742
Laura A. Coruzzi (Reg. No.)
by: Reg No 43,341

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Enclosures

PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

Inventors: Peter Nawroth
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(650) 493-4935

Attorney Docket No.: 8484-075-999

TABLE OF CONTENTS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

This is a national phase filing of the Application No. PCT/DE98/01306, which was filed with the Patent Corporation Treaty on May 8, 1998, and is entitled to priority of the German Patent Application 197 19 652.7 filed May 9, 1997.

I. FIELD OF THE INVENTION

The present invention relates to the use of tissue factor for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing.

II. BACKGROUND OF THE INVENTION

The body is provided with blood by means of blood vessels. Blood vessels comprise endothelial and smooth muscle cells. In many diseases, blood vessels and the formation thereof, respectively, are impaired. This is found, *e.g.,* in impaired wound healing as in the case of diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and infected ulcer. There are also major problems in connection with wound healing in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Also known are weak sutures and wound healing impairment in the case of operations, particularly of the intestines and transplantations of skin or other organs, respectively. Up to the present, there are no satisfactory products or means by which it is possible to take steps in the case of blood vessel diseases, in particular impaired wound healing.

Therefore, it is the object of the present invention to provide a product by means of which the above objective can be achieved.

III. SUMMARY OF THE INVENTION

The present invention relates to the use of tissue factor for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the formation of vessels (blood vessels) in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control.

FIGURE 2 shows the formation of vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tisuse factor, and (c) is a control. The blood vessels are made visible by hematoxylin/eosin staining (FIGURE 2A). In FIGURE 2B, the number of blood vessels is shown by way of diagram.

FIGURE 3 shows the presence of smooth muscle cells in newly formed vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control. The muscle cells are made visible by α-actin staining (FIGURE 3A). In FIGURE 3B, the strength of the staining is shown by way of diagram.

V. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a product by means of which the above objective can be achieved. According to the invention this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention relates to the use of tissue factor for influencing blood vessel formation, in particular for activating blood vessel formation, above all for wound healing.

The present invention is based on the applicant's finding that in wounds of animals tissue factor results in the formation of vessels (blood vessels). He found out that the vessels comprise endothelial and smooth muscle cells. The applicant also recognized that wound healing can be achieved by means of tissue factor. Furthermore, the applicant discovered that vessel formation can be prevented by inhibiting tissue factor.

Tissue factor is a transmembrane glycoprotein which binds the blood clotting factors VII and VITa, respectively. An activation of the blood clotting factors X and IX, respectively, is effected by this bond, so that the blood coagulation is started via the extrinsic path and intrinsic path, respectively. Tissue factor has a molecular weight of 43 to 46 kD. Its primary structure is known as is the gene for tissue factor and its localization on the chromosome. Scarpati *et al.*,1987, *Biochemistry* 26:5234-5238.

According to the invention tissue factor is used for activating vessel formation, particularly for wound healing. The expression "tissue factor" relates to a tissue factor of any kind and origin. It may be an animal or human tissue factor. It can be glycosylated or non-glycosylated. Also, it may be a fragment of tissue factor which is capable of forming vessels, in particular for wound healing. The tissue factor can have a wild-type sequence. Its sequence can also differ from the wild-type sequence by one or several amino acids. In addition, the tissue factor can be part of a fusion protein.

In a preferred embodiment, the tissue factor is present in the form of an expressible nucleic acid. It may be a DNA and/or RNA, a DNA, particularly a genomic or cDNA and fragments thereof, respectively, being preferred. The above statements made on the tissue factor apply here correspondingly to the nucleic acid.

The expression of the nucleic acid can be achieved as usual. It can be favorable for the nucleic acid, *e.g.,* as a DNA, particularly cDNA, to be present in a vector which is suitable for expression in animal cells. A person skilled in the art is familiar with such expression vectors. For example, they may be virus or plasmid vectors. It is advantageous for the vectors not to integrate into the genome of cells but remain episomally within the cells. By this, a transient expression of the tissue factor is achieved, which is preferred. The nucleic acid as a DNA, particularly cDNA, can also be controlled by a constitutive or inducible promoter. An inducible promoter can be, *e.g.,* tissue-, organ- and/or tumor-specific. It can be favorable for the nucleic acid as DNA, particularly cDNA, to be controlled by the CMV promoter, *e.g.,* in the expression vector pcDNA3 (Invitrogen company) or controlled by the SV4O promoter, *e.g.,* in the expression vector pSVK3 (Pharmacia company). Such expression plasmids referred to as pcDNA3-TF (tissue factor) and pSVK3-TF, respectively, also represent a subject matter of the present invention. It can be particularly advantageous for the nucleic acids as DNA, particularly cDNA, to be present

in a Sindbis virus replicon vector. Such a vector permits an extremely high expression of the nucleic acid. An example of such a vector is the ELVS vector system from Viagene Inc. An expression plasmid referred to as ELVS-TF (tissue factor) also represents a subject matter of the present invention. For the preparation of an above vector, a person skilled in the art will use known methods. Reference is made to Maniatis *et al.*, 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, by way of supplement.

According to the invention tissue factor is used for activating vessel formation, in particular for wound healing. The expression "vessel formation" relates to a vessel formation of any kind and at any site. For example, it relates to a vessel formation serving for replacing impaired, *e.g.*, old, blood vessels. They can be present, *e.g.*, in the brain or heart, so that an apoplexy or infarction can be prevented or treated. Precautions can also be taken against presbyphrenia. In addition, it relates to a vessel formation for treating arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris as well as the prevention of relapses. In particular, it relates to vessel formation and wound healing. The expression "wound healing" relates to wound healing of any kind and at any site. It can be normal and impaired wound healing. The latter is found in particular in the case of diseases, such as diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and/or infected ulcer as well as poorly healing gastric ulcer. Impaired wound healing is also found in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Impaired wound healing will also be given if weak sutures and impaired healing occur after operations, particularly of the intestines and transplantations of skin and other organs, respectively. Impaired wound healing is also found in the case of bone fractures, burns and treatments using steroids.

According to the invention tissue factor is administered in the form of a protein or an expressible nucleic acid to activate vessel formation, in particular for wound healing. It may be favorable for the tissue factor to be administered in combination with further factors supporting vessel formation, in particular for wound healing, such as vascular endothelial growth factor (VEGF). These factors can also be present in the form of proteins and/or expressible nucleic acids. The tissue factor and said factors can be administered simultaneously or successively. The kind of administration of tissue factor alone and

together with said factors, respectively, can orient itself by the site of action, *i.e.,* at the site where blood vessel formation, in particular for wound healing, shall take place. For example, it is an obvious thing to treat an area on the body surface locally and one within the interior of the body systemically. Common methods can be used for the administration of tissue factor alone and together with said factors, respectively. For the local administration it is, *e.g.,* favorable to pack the factor or factors into liposomes or absorb them onto carriers, particularly gold particles, and apply the liposomes to the corresponding site of the body and shoot the carriers, particularly gold particles, into the tissue, respectively. Furthermore, pharmaceutical compositions are provided for the administration of tissue factor alone and together with said factors, respectively, which may contain common auxiliary substances such as carriers, solvents, etc. Such compositions also represent a subject matter of the present invention.

According to the invention tissue factor is also used for inhibiting blood vessel formation. For this purpose, the tissue factor can be present in the form of an antibody inhibiting it. The tissue factor can also be present in the form of a nucleic acid which has an antisense effect on the expression of tissue factor. In particular tumoral diseases can be treated by the inhibition of vessel formation.

By means of the present invention it is possible to influence vessel formation. In particular, vessel formation can be activated. The resulting blood vessels comprise endothelial and smooth muscle cells. Thus, the present invention is suited for the prevention and treatment of the most varying diseases. Examples thereof are indicated above. In particular, the present invention is suitable for the treatment and/or prophylaxis of impaired wound healing, above all in the case of diabetes mellitus, where it is possible to heal large open wounds located at the extremities. In addition, vessel formation can be inhibited by means of the present invention. Thus, the present invention is also suited to treat diseases, such as tumoral diseases. The present invention makes a major contribution to modern medicine.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not

limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

VI. EXAMPLE

Preparation of a tissue factor-expressing plasmid and its use for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing

(A) The entire translated region (1.8 kb) of the mouse tissue factor gene was integrated into the BamHI site of the multiple cloning site of pcDNA3 (Invitrogen). Thus, this region was controlled by the CMV promoter. The expression plasmid pcDNA3-TF was obtained. In the same way, the coding region (0.7 kb) of the mouse tissue factor gene was integrated in the antisense orientation into the EcoRI site of the multiple cloning site of pcDNA3. Thus, this region was also controlled by the CMV promoter. The expression plasmid pcDNA3-TF-AS was obtained.

6 mm full thickness wounds each were placed on the backs of three female NOD mice (Bomholtgaard, Denmark) at a distance of 8 to 10 mm. These wounds were treated with mixtures containing 2 μg pcDNA3-TF (a), pcDNA3-TF-AS (b) and pcDNA3 (control (c)), respectively, and 12 μg DOTAP transfection reagent (Boehringer Mannheim) each. The wounds were covered with Ohmann Opraflex.

For proving the formation of vessels (blood vessels) in the wounds, 300 μl of ink (Nigrosin, Sigma) each were injected into the caudal vein of the mice 6 days and 8 days, respectively, following the administration of the mixtures. Thereafter, the animals were killed and the skin regions with the wounds were examined under a microscope.

It showed that if a tissue factor-expressing vector (a) is administered, vessels (blood vessels) will be formed in wounds and thus wound healing will be promoted. It also turned out that an antisense tissue factor can inhibit the formation of blood vessels.

(B) As described under (A), six NOD mice were treated. After 6 days and 8 days, respectively, the animals were killed and the corresponding skin regions were examined under a microscope after having been subjected to α-actin staining (with Sm-actin antibodies from Dianova).

It showed that the blood vessels formed comprise smooth muscle cells.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED IS:

1. Use of tissue factor or a fragment thereof for influencing blood vessel formation.

2. Use according to claim 1, wherein the influence is an activation of blood vessel formation.

3. Use according to claim 2, wherein the blood vessel formation for wound healing is concerned.

4. Use according to claim 2 or 3, wherein the wound healing in the case of diabetis mellitus, vasculitis, arterial conclusive disease, chronic venous and infected ulcer, innervation impairment, decubital gangrene and weak sutures in the case of operations is concerned.

5. Use according to claim 2 or 3, wherein the blood vessel formation in the case of arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep vnous thrombosis of the legs/ulcus cruris is concerned.

6. Use according to claim 2, wherein the blood vessel formation for replacing impaired blood vessels is concerned.

7. Use according to any one of claims 1 to 6, wherein the tissue factor or a fragment thereof is present as expressible nucleic acid.

8. Use according to claim 7, wherein the expression of the nucleic acid is transient.

9. Use according to claim 7 or 8, wherein the nucleic acid is a DNA.

10. Use according to any one of claims 7 to 9, wherein the nucleic acid is controlled by a constitutive or inducible promoter.

11. Use according to any one of claims 7 to 10, wherein the nucleic acid is present in a Sindbis virus replicon vector.

12. Use according to any one of claims 7 to 10, wherein the nucleic acid is controlled by a CMV or 5V40 promoter.

13. Use according to any one of claims 1 to 12, wherein the tissue factor is present in a liposome or on a carrier, particularly gold particle.

14. Use according to any one of claims 1 to 13, wherein the tissue factor is present in combination with further factors promoting the formation of blood vessels.

15. Use according to claim 14, wherein the factors are present as expressible nucleic acids.

16. Use according to claim 14 or 15, wherein one of the factors is VEGF.

17. Use according to any one of claims 1 to 16, wherein the tissue factor is present in a pharmaceutical composition.

18. Use according to claim 1, wherein the influence is an inhibition of blood vessel formation.

19. Use according to claim 18, wherein the tissue factor is present in the form of an antibody inhibiting it.

20. Use according to claim 18, wherein the tissue factor is present in the form of a nucleic acid, which has an antisense effect on the expression of tissue factor.

21. Use according to any one of claims 18 to 20, wherein the tissue formation is inhibited in a tumoral disease.

CLAIMS AS AMENDED UNDER ARTICLE 34

1. Use of tissue factor or a fragment thereof for the well-calculated therapeutic influence of blood vessel formation by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

2. Use according to claim 1, wherein the influence is an activation of blood vessel formation.

3. Use of a tissue factor or a fragment thereof for influencing the wound healing by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

4. Use according to claim 3, wherein the wound healing in the case of diabetis mellitus, vasculitis, arterial conclusive disease, chronic venous and infected ulcer, innervation impairment, decubital gangrene and weak sutures in the case of operations is concerned.

5. Use according to claim 1 or 2, wherein the blood vessel formation in the case of arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris is concerned.

6. Use according to claim 1 or 2, wherein the blood vessel formation for replacing impaired blood vessels is concerned.

7. Use according to any one of claims 1 to 6, wherein the tissue factor or a fragment thereof is present as expressible nucleic acid.

8. Use according to claim 7, wherein the expression of the nucleic acid is transient.

9. Use according to claim 7 or 8, wherein the nucleic acid is a DNA.

10. Use according to any one of claims 7 to 9, wherein the nucleic acid is controlled by a constitutive or indicuble promoter.

11. Use according to any one of claims 7 to 10, wherein the nucleic acid is present in a Sindbis virus replicon vector.

12. Use according to any one of claims 7 to 10, wherein the nucleic acid is controlled by a CMV or 5V40 promoter.

13. Use according to any one of claims 1 to 12, wherein the tissue factor is present in a liposome or on a carrier, particularly gold particle.

14. Use according to any one of claims 1 to 13, wherein the tissue factor is present in combination with further factors promoting the formation of blood vessels.

15. Use according to claim 14, wherein the factors are present as expressible nucleic acids or functional proteins.

16. Use according to claim 14 or 15, wherein one of the factors is VEGF.

17. Use according to any one of claims 1 to 16, wherein the tissue factor is present in a pharmaceutical composition.

ABSTRACT

The present invention relates to the use of tissue factor for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing.

M 4297

Tissue Factor for Influencing Blood Vessel Formation

The present invention relates to the use of tissue factor for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing.

The body is provided with blood by means of blood vessels. Blood vessels comprise endothelial and smooth muscle cells. In many diseases, blood vessels and the formation thereof, respectively, are impaired. This is found e.g. in impaired wound healing as in the case of diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and infected ulcer. There are also major problems in connection with wound healing in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Also known are weak sutures and wound healing impairment in the case of operations, particularly of the intestines and transplantations of skin or other organs, respectively. Up to the present, there are no satisfactory products or means by which it is possible to take steps in the case of blood vessel diseases, in particular impaired wound healing.

Therefore, it is the object of the present invention to provide a product by means of which the above objective can be achieved.

According to the invention this is achieved by the subject matters defined in the claims.

Thus, the subject matter of the present invention relates to the use of tissue factor for influencing blood vessel formation, in particular for activating blood vessel formation, above all for wound healing.

The present invention is based on the applicant's finding that in wounds of animals tissue factor results in the formation of vessels (blood vessels). He found out that the vessels comprise endothelial and smooth muscle cells. He also recognized that wound healing can be achieved by means of tissue factor. Furthermore, he discovered that vessel formation can be prevented by inhibiting tissue factor.

Tissue factor is a transmembrane glycoprotein which binds the blood clotting factors VII and VIIa, respectively. An activation of the blood clotting factors X and IX, respectively, is effected by this bond, so that the blood coagulation is started via the extrinsic path and intrinsic path, respectively. Tissue factor has a molecular weight of 43 to 46 kD. Its primary structure is known as is the gene for tissue factor and its localization on the chromosome (cf. Scarpati, E.M., et al., Biochemistry 26, (1987), 5234-5238).

According to the invention tissue factor is used for activating vessel formation, particularly for wound healing. The expression "tissue factor" relates to a tissue factor of any kind and origin. It may be an animal or human tissue factor. It can be glycosylated or non-glycosylated. Also, it may be a fragment of tissue factor which is capable of forming vessels, in particular for wound healing. The tissue factor can have a wild-type sequence. Its sequence can also differ from the wild-type sequence by one or several amino acids. In addition, the tissue factor can be part of a fusion protein.

In a preferred embodiment, the tissue factor is present in the form of an expressible nucleic acid. It may be a DNA and/or RNA, a DNA, particularly a genomic or cDNA and fragments thereof, respectively, being preferred. The above statements made on the tissue factor apply here correspondingly to the nucleic acid.

The expression of the nucleic acid can be achieved as usual. It can be favorable for the nucleic acid, e.g. as a DNA, particularly cDNA, to be present in a vector which is suitable for expression in animal cells. A person skilled in the art is familiar with such expression vectors. For example, they may be virus or plasmid vectors. It is advantageous for the vectors not to integrate into the genome of cells but remain episomally within the cells. By this, a transient expression of the tissue factor is achieved, which is preferred. The nucleic acid as a DNA, particularly cDNA, can also be controlled by a constitutive or inducible promoter. An inducible promoter can be e.g. tissue-, organ- and/or tumor-specific. It can be favorable for the nucleic acid as DNA, particularly cDNA, to be controlled by the CMV promoter e.g. in the expression vector pcDNA3 (Invitrogen company) or controlled by the SV40 promoter, e.g. in the expression vector pSVK3 (Pharmacia company). Such expression plasmids referred to as pcDNA3-TF (tissue factor) and pSVK3-TF, respectively, also represent a subject matter of the present invention. It can be particularly advantageous for the nucleic acids as DNA, particularly cDNA, to be present in a Sindbis virus replicon vector. Such a vector permits an extremely high expression of the nucleic acid. An example of such a vector is the ELVS vector system from Viagene Inc. An expression plasmid referred to as ELVS-TF (tissue factor) also represents a subject matter of the present invention. For the preparation of an above vector, a person skilled in the art will use known methods. Reference is made to Maniatis, T., et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, by way of supplement.

According to the invention tissue factor is used for activating vessel formation, in particular for wound healing. The expression "vessel formation" relates to a vessel formation of any kind and at any site. For example, it relates to a vessel formation serving for replacing impaired, e.g. old, blood vessels. They can be present e.g. in the brain or heart, so that an apoplexy or infarction can

be prevented or treated. Precautions can also be taken against presbyphrenia. In addition, it relates to a vessel formation for treating arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris as well as the prevention of relapses. In particular, it relates to vessel formation and wound healing. The expression "wound healing" relates to wound healing of any kind and at any site. It can be normal and impaired wound healing. The latter is found in particular in the case of diseases, such as diabetes mellitus, vasculitis, arterial occlusive disease, chronic venous and/or infected ulcer as well as poorly healing gastric ulcer. Impaired wound healing is also found in the case of innervation impairment such as paraplegia, leprosy, neuropathy, etc., and decubital gangrene of persons in need of care. Impaired wound healing will also be given if weak sutures and impaired healing occur after operations, particularly of the intestines and transplantations of skin and other organs, respectively. Impaired wound healing is also found in the case of bone fractures, burns and treatments using steroids.

According to the invention tissue factor is administered in the form of a protein or an expressible nucleic acid to activate vessel formation, in particular for wound healing. It may be favorable for the tissue factor to be administered in combination with further factors supporting vessel formation, in particular for wound healing, such as vascular endothelial growth factor (VEGF). These factors can also be present in the form of proteins and/or expressible nucleic acids. The tissue factor and said factors can be administered simultaneously or successively. The kind of administration of tissue factor alone and together with said factors, respectively, can orient itself by the site of action, i.e. at the site where blood vessel formation, in particular for wound healing, shall take place. For example, it is an obvious thing to treat an area on the body surface locally and one within the interior of the body systemically. Common methods can be used for the

administration of tissue factor alone and together with said factors, respectively. For the local administration it is e.g. favorable to pack the factor or factors into liposomes or absorb them onto carriers, particularly gold particles, and apply the liposomes to the corresponding site of the body and shoot the carriers, particularly gold particles, into the tissue, respectively. Furthermore, pharmaceutical compositions are provided for the administration of tissue factor alone and together with said factors, respectively, which may contain common auxiliary substances such as carriers, solvents, etc. Such compositions also represent a subject matter of the present invention.

According to the invention tissue factor is also used for inhibiting blood vessel formation. For this purpose, the tissue factor can be present in the form of an antibody inhibiting it. The tissue factor can also be present in the form of a nucleic acid which has an antisense effect on the expression of tissue factor. In particular tumoral diseases can be treated by the inhibition of vessel formation.

By means of the present invention it is possible to influence vessel formation. In particular, vessel formation can be activated. The resulting blood vessels comprise endothelial and smooth muscle cells. Thus, the present invention is suited for the prevention and treatment of the most varying diseases. Examples thereof are indicated above. In particular, the present invention is suitable for the treatment and/or prophylaxis of impaired wound healing, above all in the case of diabetes mellitus, where it is possible to heal large open wounds located at the extremities. In addition, vessel formation can be inhibited by means of the present invention. Thus, the present invention is also suited to treat diseases, such as tumoral diseases. The present invention makes a major contribution to modern medicine.

Brief description of the drawing

Figure 1 shows the formation of vessels (blood vessels) in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control.

Figure 2 shows the formation of vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tisuse factor, and (c) is a control. The blood vessels are made visible by hematoxylin/eosin staining (figure 2A). In figure 2B, the number of blood vessels is shown by way of diagram.

Figure 3 shows the presence of smooth muscle cells in newly formed vessels in wounds transfected with a tissue factor-expressing vector (a). (b) is a vector which codes for an antisense tissue factor, and (c) is a control. The muscle cells are made visible by α-actin staining (figure 3A). In figure 3B, the strength of the staining is shown by way of diagram.

The present invention is explained by the example.

Example: Preparation of a tissue factor-expressing plasmid and its use for influencing blood vessel formation, particularly for activating blood vessel formation, above all for wound healing

(A) The entire translated region (1.8 kb) of the mouse tissue factor gene was integrated into the BamHI site of the multiple cloning site of pcDNA3 (Invitrogen). Thus, this region was controlled by the CMV promoter. The expression plasmid pcDNA3-TF was obtained. In the same way, the coding region

(0.7 kb) of the mouse tissue factor gene was integrated in the antisense orientation into the EcoRI site of the multiple cloning site of pcDNA3. Thus, this region was also controlled by the CMV promoter. The expression plasmid pcDNA3-TF-AS was obtained.

6 mm full thickness wounds each were placed on the backs of three female NOD mice (Bomholtgaard, Denmark) at a distance of 8 to 10 mm. These wounds were treated with mixtures containing 2 µg pcDNA3-TF (a), pcDNA3-TF-AS (b) and pcDNA3 (control (c)), respectively, and 12 µg DOTAP transfection reagent (Boehringer Mannheim) each. The wounds were covered with Ohmann Opraflex.

For proving the formation of vessels (blood vessels) in the wounds, 300 µl of ink (Nigrosin, Sigma) each were injected into the caudal vein of the mice 6 days and 8 days, respectively, following the administration of the mixtures. Thereafter, the animals were killed and the skin regions with the wounds were examined under a microscope.

It showed that if a tissue factor-expressing vector (a) is administered, vessels (blood vessels) will be formed in wounds and thus wound healing will be promoted. It also turned out that an antisense tissue factor can inhibit the formation of blood vessels.

(B) As described under (A), six NOD mice were treated. After 6 days and 8 days, respectively, the animals were killed and the corresponding skin regions were examined under a microscope after having been subjected to α-actin staining (with Sm-actin antibodies from Dianova).

It showed that the blood vessels formed comprise smooth muscle cells.

ARTICLE 34

M 4297

Amended Claims

1. Use of tissue factor or a fragment thereof for the well-calculated therapeutic influence of blood vessel formation by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

2. Use according to claim 1, wherein the influence is an activation of blood vessel formation.

3. Use of a tissue factor or a fragment thereof for influencing the wound healing by induction of a local expression of a tissue factor nucleic acid or by local application of a functional tissue factor protein.

4. Use according to claim 3, wherein the wound healing in the case of diabetis mellitus, vasculitis, arterial conclusive disease, chronic venous and infected ulcer, innervation impairment, decubital gangrene and weak sutures in the case of operations is concerned.

5. Use according to claim 1 or 2, wherein the blood vessel formation in the case of arteriosclerosis, Crohn's disease and ulcerative colitis, diabetic retinopathy and deep venous thrombosis of the legs/ulcus cruris is concerned.

6. Use according to claim 1 or 2, wherein the blood vessel formation for replacing impaired blood vessels is concerned.

7. Use according to any one of claims 1 to 6, wherein the tissue factor or a fragment thereof is present as expressible nucleic acid.

8. Use according to claim 7, wherein the expression of the nucleic acid is transient.

9. Use according to claim 7 or 8, wherein the nucleic acid is a DNA.

10. Use according to any one of claims 7 to 9, wherein the nucleic acid is controlled by a constitutive or indicuble promoter.

11. Use according to any one of claims 7 to 10, wherein the nucleic acid is present in a Sindbis virus replicon vector.

12. Use according to any one of claims 7 to 10, wherein the nucleic acid is controlled by a CMV or SV40 promoter.

13. Use according to any one of claims 1 to 12, wherein the tissue factor is present in a liposome or on a carrier, particularly gold particle.

14. Use according to any one of claims 1 to 13, wherein the tissue factor is present in combination with further factors promoting the formation of blood vessels.

15. Use according to claim 14, wherein the factors are present as expressible nucleic acids or functional proteins.

16. Use according to claim 14 or 15, wherein one of the factors is VEGF.

17. Use according to any one of claims 1 to 16, wherein the tissue factor is present in a pharmaceutical composition.

Abstract of the Disclosure

Tissue Factor for Influencing Blood Vessel Formation

The present invention relates to the use of tissue factor for influencing blood vessel formation, in particular for activating blood vessel formation, above all for wound healing.

(a)

(b)

(c)

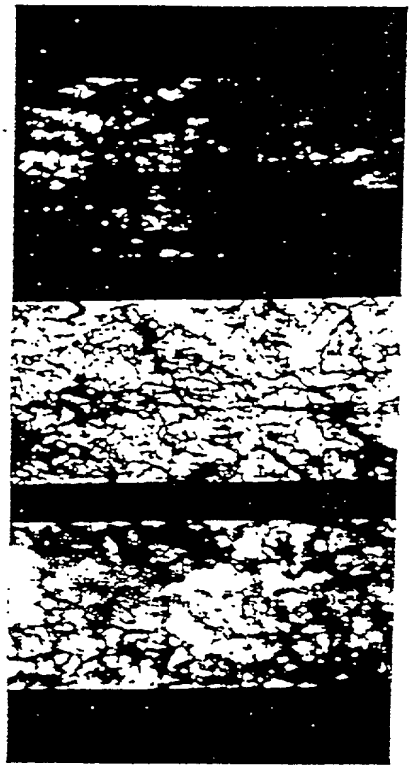
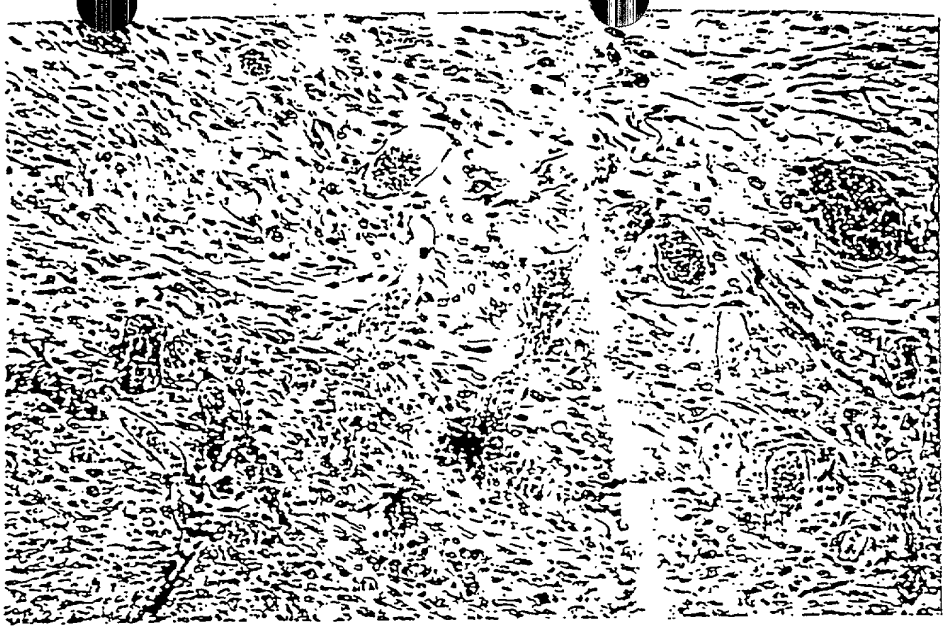


Fig. 1

(a)



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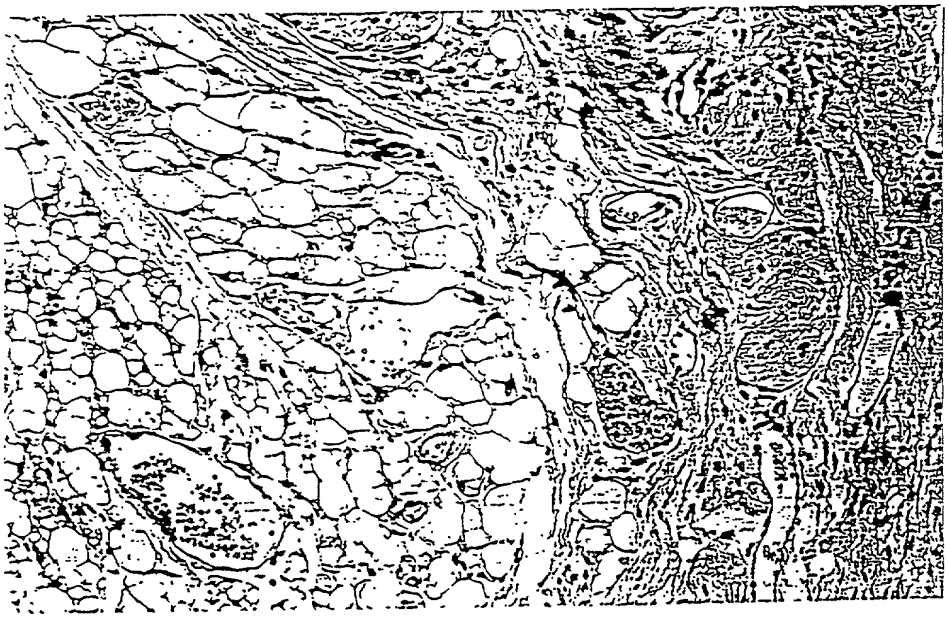


(b)

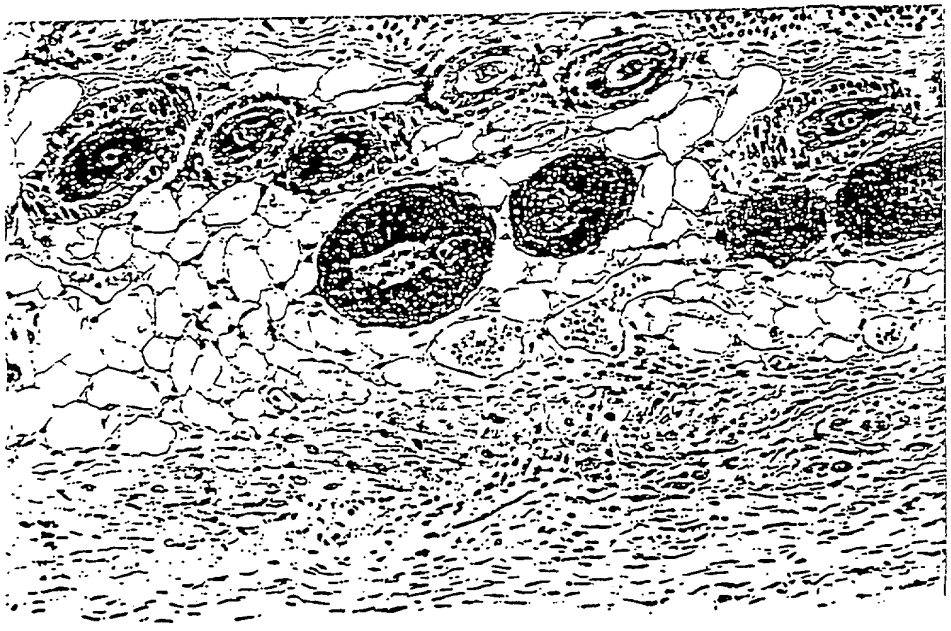


Fig. 2A

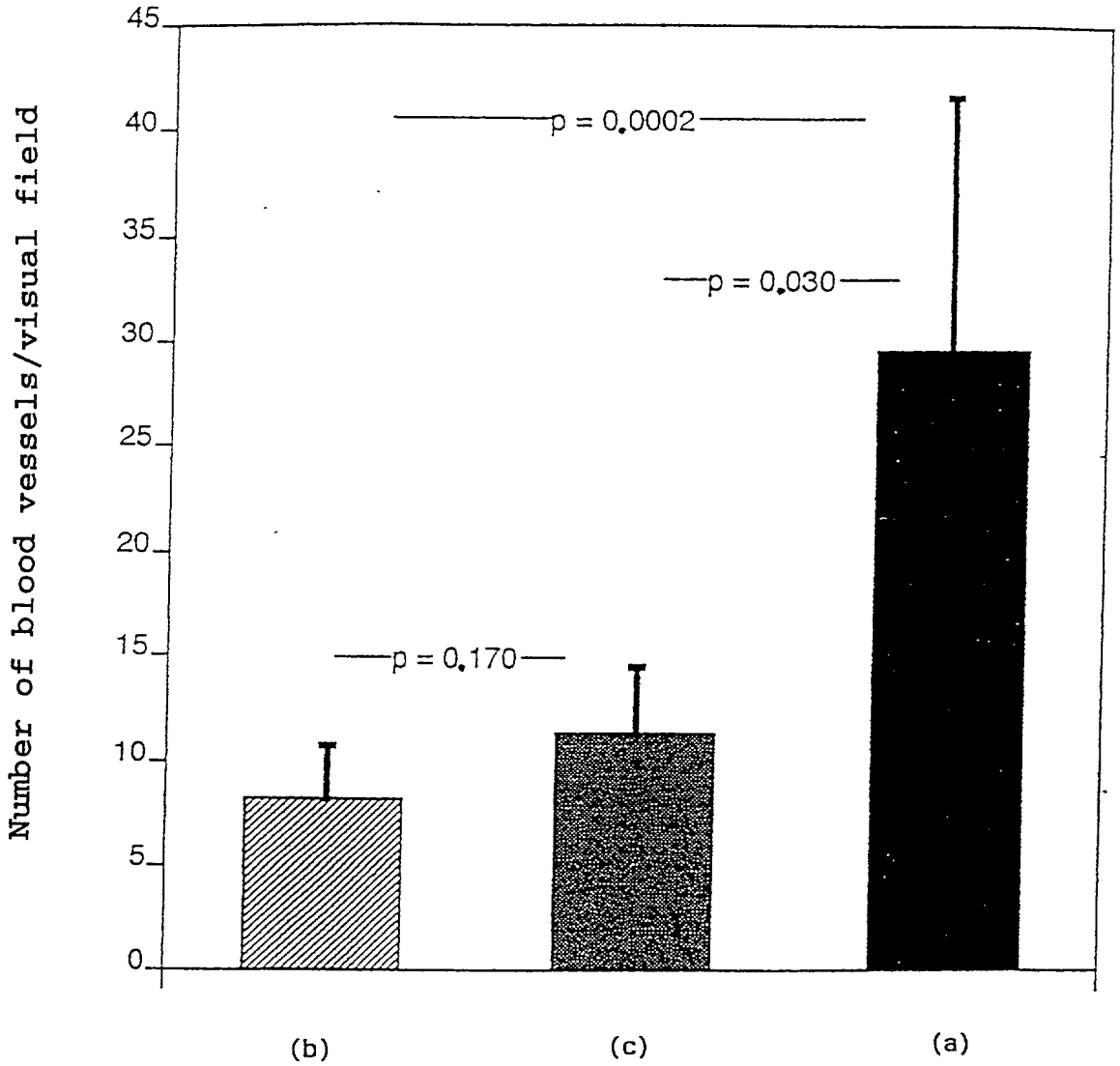
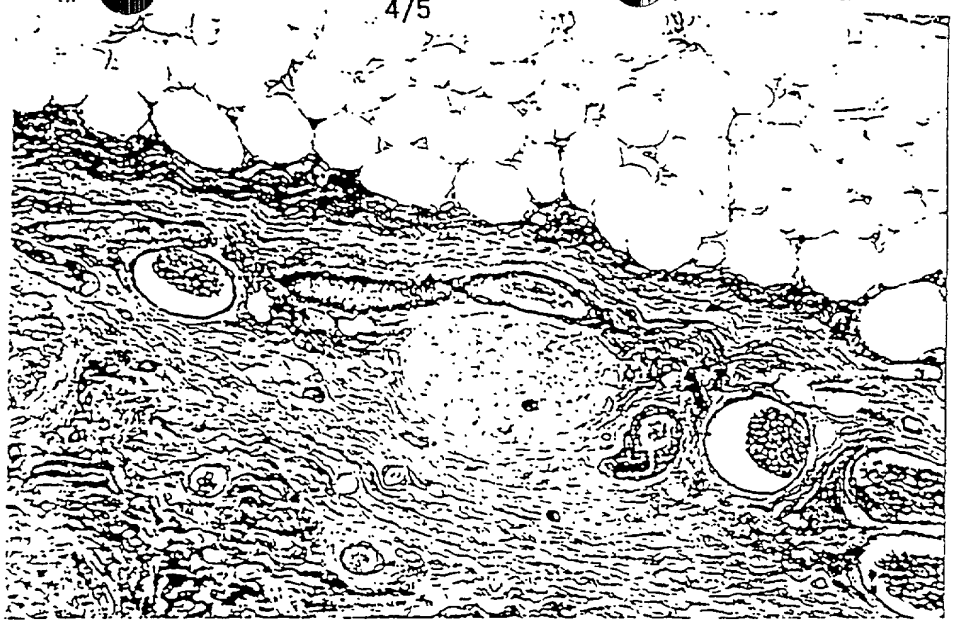
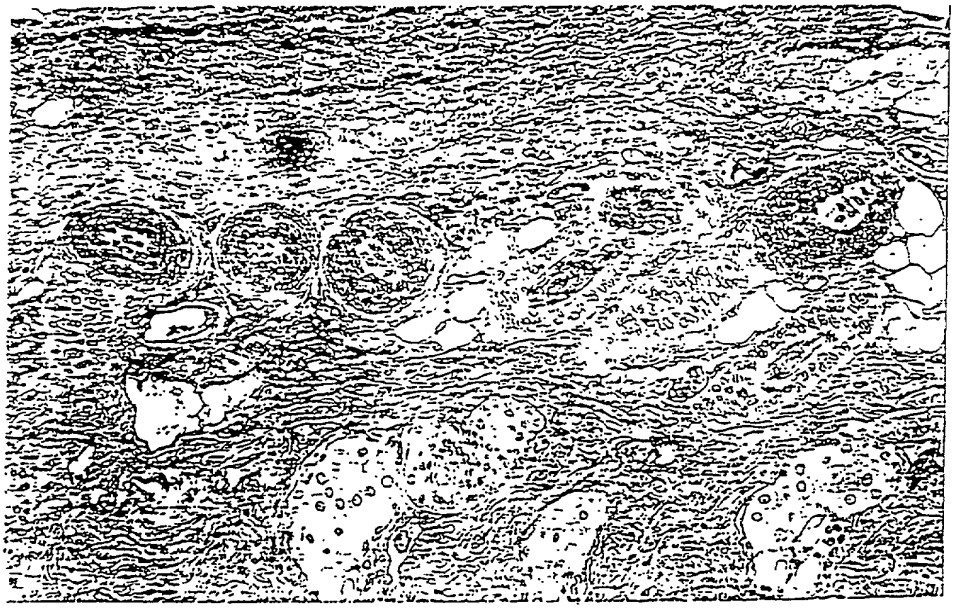

Number of blood vessels/visual field
45
40
35
30
25
20
15
10
5
0
p = 0.0002
p = 0.030
p = 0.170
(b)
(c)
(a)

Fig. 2B

(a)



(c)



(b)

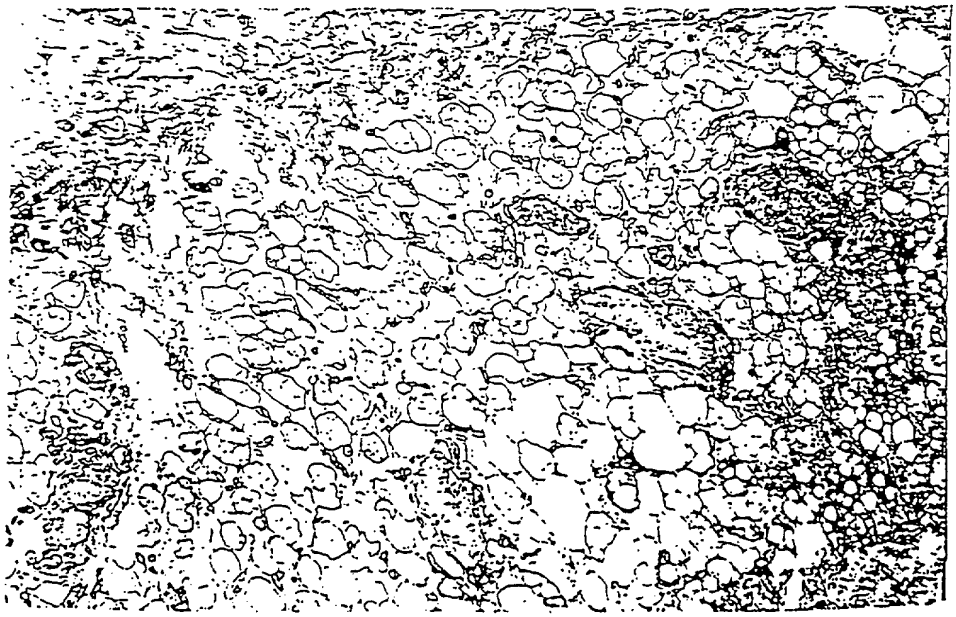
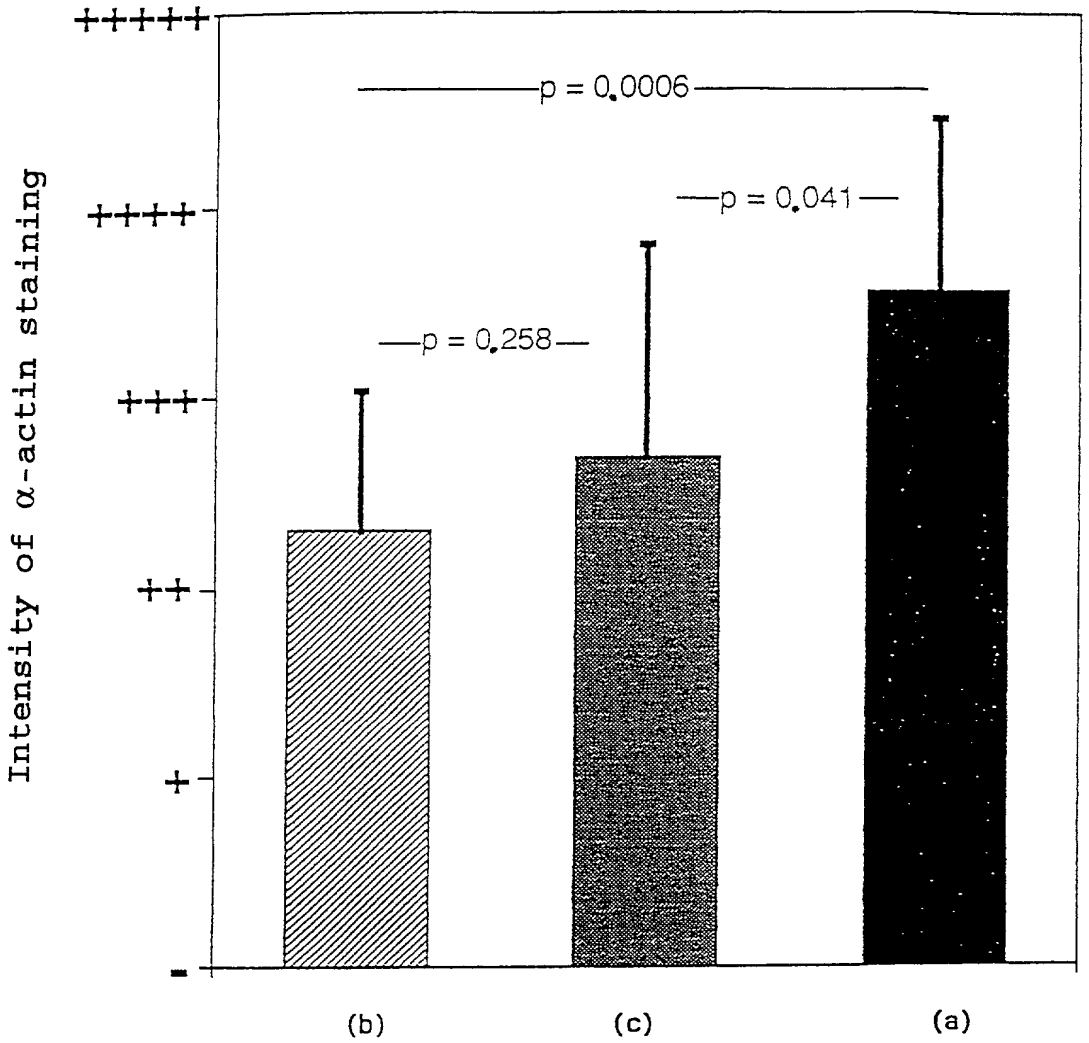


Fig. 3A



Graduation of α-actin staining:

-	=	no staining
+	=	weak staining in individual regions of the vessel walls
++	=	weak staining of the entire vessel
+++	=	partly weak, partly strong staining of the vessel wall
++++	=	strong staining of the entire vessel wall
+++++	=	very strong staining in all regions of the vessel

Fig. 3B

DECLARATION
AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

TISSUE FACTOR FOR INFLUENCING BLOOD VESSEL FORMATION

and for which a patent application:
□ is attached hereto and includes amendment(s) filed on *(if applicable)*
☒ was filed in the United States on November 9, 1999 as Application No. 09/423,712 *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. PCT/DE98/01306 on May 8, 1998 and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
PCT/DE98/01306	PCT	May 8, 1998	YES ☒ NO □
19719652.7	Germany	May 9, 1997	YES ☒ NO □

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

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205	FULL NAME OF INVENTOR	LAST NAME:	FIRST NAME:	MIDDLE NAME:	
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	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:	
	POST OFFICE ADDRESS	STREET:	CITY:	STATE OR COUNTRY:	ZIP CODE:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

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DATE: 25.07.00	DATE: March 15, 2000	DATE: 01.03.2000
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DATE	DATE	DATE